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C.L. Schultz, B. Kaufman, D. Hamilton, A. Hartman, M. Ruiz, C. Powell, and S. Berman

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Cell wall structures which may be important for successful immunization with *Salmonella–Shigella* hybrid vaccines

C.L. Schultz*, B. Kaufman‡, D. Hamilton§, A. Hartman*, M. Ruiz†, C. Powell* and S. Berman*

Three separate lots of* S. typhi/S. sonnei *hybrid (Ty/Shig) live oral vaccine strain 5076-1C were tested for efficacy in human volunteers challenged with virulent* S. sonnei. *Two lots (2 and 5) protected volunteers, a third lot (8) did not. The three lots were evaluated by immunological tests and electron microscopy. Lots 2 and 5, which protected, contained bacteria that reacted with anti-flagellar serum and had observable attached flagella and pili. Lot 8, which failed to protect, did not react with anti-flagellar serum, and had no observable pili. There was no correlation between vaccine efficacy and the reaction of IgG in patient's sera in western blot analysis. Surface structures on the Ty-Shig hybrid may be important for generating a protective immune response.

Keywords: *Salmonella Shigella* hybrid vaccine; immunization; cell wall structures

Introduction

A live-oral vaccine for typhoid fever has been developed which utilizes a galactose epimeraseless (gal E) mutant of *Salmonella typhi* Ty21a. This vaccine has shown promise in prevention of typhoid in humans[1-4]. Vaccination against *Shigella sonnei* may be conferred by oral administration of a *Salmonella typhi Shigella sonnei* (5076-1C) (Ty Shig) hybrid vaccine[2]. This hybrid vaccine consists of an *S. typhi* Ty21a host cell, into which is inserted a 120 kDa plasmid carrying genes for the *S. sonnei* 53 G Form 1 somatic antigen and for the biological processing of lactose on MacConkey Agar[6]. The *Salmonella* host cell will express these somatic antigens.

Two lots of vaccine, Lot 2 (1982) and Lot 5 (1984), protected humans against challenge with a virulent strain of *Shigella sonnei* (53G)[2]. However, a third lot of vaccine, Lot 8, which was produced in 1986, failed to protect human volunteers against challenge[2]. Prior to use the three vaccines were assayed for numbers of organisms, percentage lactose positive colonies (on MacConkey Agar), reaction against *Salmonella* and *Shigella* antisera, and protective immunization of mice. After the failure of the Lot 8 vaccine in humans, the preparations were subsequently assayed by additional tests to determine if there were any characteristics which showed a positive correlation with the results of human vaccine trials. Electron microscopic observations of negatively stained preparations of the three vaccine lots were conducted, which demonstrated differences in the external structures on organisms comprising the vaccines. Furthermore, there were differences in the *Salmonella* flagellar H antigen agglutination reaction. Reported here are the similarities and differences between these vaccine lots, and a suggested explanation for the failure of the Lot 8 vaccine to protect humans against challenge.

*Department of Biologics Research, †Department of Immunology, Walter Reed Army Institute of Research, Washington D.C. 20307-5100, USA. ‡Genex Corporation, Gaithersburg, MD 20877, USA. §Department of Microbiology, University of Maryland, College Park, Maryland 22807, USA.
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Materials and methods

Vaccine seeds

The Lot 2 and Lot 5 master seeds were obtained from S. Formal (WRAIR). The master seed for the Lot 8 vaccine candidate was obtained by rehydrating a bottle of freeze-dried, Lot 5 vaccine and passing the outgrowth on MacConkey Agar. This was necessary because over time the plasmid became unstable and an increased rate of spontaneous segregation occurred. Individual lactose positive colonies were passaged eight times on MacConkey Agar in an attempt to select a stable isolate, that is, an isolate in which the plasmid would segregate at a reduced frequency. Lactose positive reactions of MacConkey Agar were used as a marker to indicate plasmid presence. At passage eight, all isolated colonies were lactose positive. The master seed bacteria were harvested from the pass 8 MacConkey Agar plate, diluted in sucrose (7.5%)-phosphate (0.004 M KH_2PO_4 and K_2HPO_4 0.007 M)-glutamate (0.0044 M monosodium glutamate) (SPG), freeze-dried, and stored at −60 C.

Vaccine production

The production scheme used to prepare the hybrid Ty Shig vaccines is shown in *Figure 1*. The master seeds for Lots 2 and 5 were rehydrated with saline, streaked on to MacConkey Agar and incubated at 35 C. After 28–36 h, about a dozen lactose positive colonies were picked,

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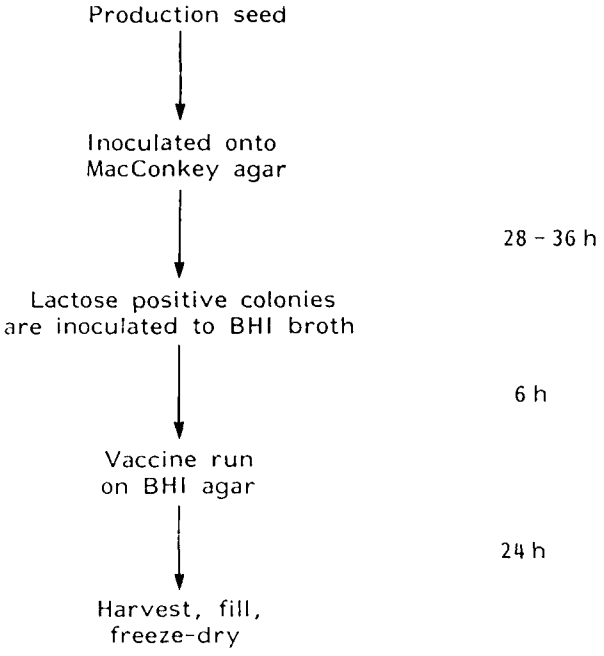

Figure 1 Production scheme for typhoid *Shigella* hybrid vaccines

inoculated into Brain-Heart Infusion (BHI) Broth (Difco Laboratories, Detroit, MI) and incubated at 35 C for 6 h. After this incubation, the pooled outgrowth of bacteria was then re-inoculated into Kolle flasks (2 ml per flask) containing BHI Agar (BHIA) and incubation was continued for 18 h. The vaccine was harvested by vacuum rake into SPG as the cryopreservative. The vaccine was filled into 50 ml vaccine bottles (PGC Scientific, Gaithersburg, MD) at 5 ml bottle and frozen at −60 C in preparation for freeze-drying.

The Lot 8 vaccine was produced by rehydrating a bottle of freeze-dried Lot 8 master seed with saline, inoculating MacConkey Agar, and subsequently BHIB as described above. However, after the 6 h incubation period, the bacteria were inoculated on to NUNC phage plates (PGC Scientific, Gaithersburg, MD) containing BHIA. After 18 h incubation at 35 C, the bacteria were harvested into a Waring blender cup containing SPG and blended for two, three-second pulses. The vaccine was then bottled as described.

Pre-freeze-dry vaccine plate counts were determined on the wet product using both BHIA and MacConkey Agar as growth media. Plate counts of the freeze-dried vaccine and the percentage of organisms which were recovered from freeze-drying were determined using BHIA. The numbers of organisms carrying the *S. sonnei* plasmid were determined using MacConkey Agar. Those organisms which were lactose positive, and thus carried the plasmid, were red; those not carrying the plasmid or carrying the lactose negative form of the plasmid were pink or white. Vaccine lots which had $\geq 90\%$ of lactose positive colonies were considered suitable for testing in volunteers.

Serology

Qualitative determinations for the presence of *S. typhi* and *S. sonnei* somatic antigens were determined by slide agglutination assays using specific rabbit antisera. The flagellar H antigen reaction was titrated on the final products using a constant dilution (1:20 in physiological saline) of antisera prepared in rabbits. Serial twofold dilutions of 50 μl of the vaccine were prepared in saline on a glass slide and reacted with a 50 μl of antisera. Results were expressed as the reciprocal of the highest dilution of vaccine which resulted in agglutination.

Freeze-drying of hybrid vaccines

The freeze-drying cycle was begun at −46 C in a Stokes Model 24 PV freeze-dryer. Once vacuum was applied to the chamber, the temperature increased to −24 C over the next 8 h, and the cycle was continued at this temperature for another 14 h. The freeze-dryer was then manually adjusted to 0 C; with an increase in temperature to 0 C over the next 3 h. The cycle was continued for another 21 h. The products were stoppered under vacuum and sealed, labelled and stored at −60 C.

Bacterial examination by electron microscopy

All vaccines were prepared for electron microscopic examination in the same manner. Vaccines were first resuspended in a volume of physiological saline following the instructions on the vials. Broth (18 h, 36 C) grown cultures were washed once with physiological saline. An aliquot was then removed and placed on a clean strip of Parafilm to form a drop. A carbon coated, formvar grid was floated on the suspension for ≈ 1 min. The grids were then washed on drops of phosphate buffered saline (PBS) and then negatively stained with phosphotungstic acid (1% solution) for 1 min. The stained grids were observed in a JOEL 100 CX electron microscope. Calculations of the percentages of piliated and non-piliated bacteria were based on at least 200 organisms per observed field.

Mouse protection assays

Viable cells of the vaccine candidates were used to immunize mice. The dried hybrid vaccines lots 2, 5 and 8 were rehydrated with PBS and diluted to contain $5 \times 10^{-}$ viable cells per 0.5 ml. Outbred mice (ICR) weighing 13–16 g were inoculated intraperitoneally (IP) with 0.5 ml of diluted suspensions. Mice inoculated with 0.5 ml of saline served as controls. Immunized and control mice were challenged IP 4 weeks postimmunization with viable cells of *S. typhi* Ty2 and *S. sonnei* 53G suspended in 5.0% hog gastric mucin. Challenge suspensions contained $\approx 5.0 \times 10^2$ cells/0.5 ml for the *S. sonnei* strain and 5×10^4/0.5 ml for the *S. typhi* challenge. Deaths were recorded 72 h post challenge.

Plasmid preparation and restriction enzyme digestion analysis

Shigella sonnei single colony isolates were tested for the production of the Form 1 antigen using specific antisera. Lactose positive colonies were identified as described above[2]. Positive isolates were grown overnight using BHI broth.

Large scale isolation of the plasmid in the vaccine strain was carried out using the method of Cassie *et al.* and purifed by CsCl-ethidium bromide density gradient ultracentrifugation[3]. Smaller preparations were utilized when single colonies were screened. In these preparations a phenol:chloroform (1:1) extraction and ethanol precipitation replaced the CsCl centrifugation step.

Table 1 Comparison of three typhoid-*Shigella* vaccine lots. The assays numbered 1–7 were performed before the Lot 8 challenge study in human volunteers. The assays numbered 8 and 9 were performed after that study

	Lot 2 (9–82)	Lot 5 (1–84)	Lot 8 (2–86)
1 Fluid count (cells/ml)	1.3×10^{10}	1.5×10^{10}	1.7×10^{10}
2 Freeze-dry count (cells/ml)	7.5×10^{9}	7.8×10^{9}	4.1×10^{9}
3 Per cent recovery after freeze-drying	57	52	24
4 Per cent lactose pos.	100	90	99
5 Agglutatination with *S. typhi* antisera	2+	2+	3+
6 Agglutination with Shigella Form I antisera	4+	4+	4+
7 Mouse potency assay	Protects	Protects	Protects
8 Agglutination with H antisera	8	16	+/−
9 Electron micrographs:			
a Flagella	+	+	+
b Pili	+	+	−

Figure 2 Electron micrographs of negatively stained vaccines. Negative stained samples of (a) Lot 5, (b) Lot 2, and (c) Lot 8 were prepared as described. The preparations from Lot 5 and Lot 2 revealed fimbriae (common pili) on ≈30–40% of the bacteria. These structures were not seen on the bacteria from Lot 8. Flagella appeared in all preparations. Bar = 0.5 μm

Isolated plasmid DNA was digested with Bgl II (Life Technologies Inc., Gaithersburg, MD) and Sac I (International Biotechnologies Inc., New Haven, CT) under conditions specified by the manufacturers. The digested DNA was electrophoresed on 0.6% agarose gels containing ethidium bromide (10 μg of a 10 mg ml^{-1} solution for a 100 ml gel) in Tris-borate buffer (10× solution contains 1 M Tris Base, 0.83 M boric acid, and 10 mM EDTA) at 25 V for 12–15 h.

Electrophoresis and western blotting

Polyacrylamide gel electrophoresis (PAGE) of whole organisms was carried out essentially by the method of Laemmli[9]. Western blots were done as described by Towbin, with modifications by Sidberry *et al.* using goat anti-human IgG as the second antibody[14,15]. The lanes were purposely overloaded in an attempt to detect minor differences among the vaccine strains either by protein straining or by western blot.

Results

The three vaccine lots are compared in *Table 1*. Pre-freeze-dry counts, postfreeze-dry counts and the per cent lactose positive organisms were approximately the same for the three lots of vaccine. However, the Lot 8 vaccine had a recovery rate of about half of Lots 2 and 5. All three vaccine candidates reacted well with *S. typhi* 0 and *S. sonnei* phase 1 antisera. All three lots of vaccine had flagella (*Figure 2*). The H antigen reactions of Lots 2 and 5, were 8 and 16 respectively (*Table 1*). Although flagella were observed on bacteria in Lot 8, this vaccine preparation was not agglutinated by the typhoid flagellar H antiserum. Additionally, no pili were observed on the Lot 8 vaccine organisms. Approximately 30–40% of the bacteria of Lots 2 and 5 had pili (*Figure 2*). These results are based on calculations with at least 200 bacteria per observed field.

Since the vaccine strain contains a plasmid which is essential for the development of the immune response against *S. sonnei*, we attempted to determine if there were differences in the plasmids contained in the three vaccine lots by restriction analysis. Individual colonies of the Ty–Shig vaccine lots were scored for the lactose phenotype and expression of *S. sonnei* Form 1 antigen. However, restriction analysis of DNA from bacteria contained in the vaccine lots showed two types of colonies: (1) lactose positive, Form 1 antigen positive and (2) lactose negative. Lots 2 and 5 contained both forms and more than 80% of the individual colonies tested were both lactose positive and Form 1 antigen positive. Lot 8 contained greater than 90% of the lactose positive and Form 1 antigen positive colonies. Lactose negative colonies, which are white on MacConkey Agar, appear to have undergone a substantial deletion of plasmid material. There were no differences between plasmid forms isolated from bacteria taken from the three vaccine lots.

Figure 3 shows SDS-PAGE profiles of vaccine Lots 2, 5 and 8. There were no observable differences between the protective Lots 2 and 5 and the non-protective Lot 8. This was true whether the samples were run under reducing or non-reducing conditions on overloaded gels (*Figure 3*). The similarly treated parent *S. sonnei* 53G strain demonstrated no observable differences in the protein profile.

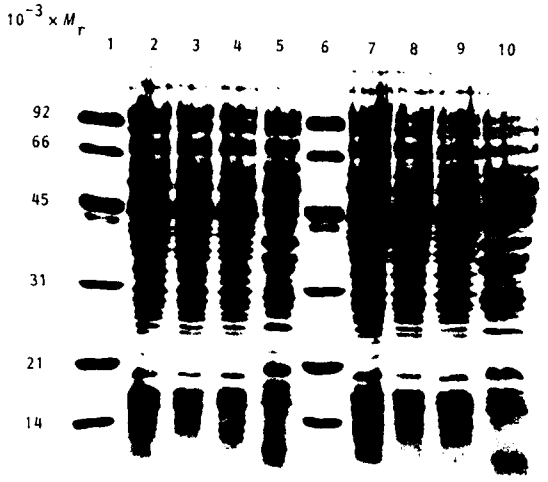

Figure 3 Acrylamide gel profiles of reconstituted vaccine lots. Samples were separated on 12% polyacrylamide gels in the presence of SDS and stained with Coomassie blue. Samples in lanes 1-6 were also treated with mercaptoethanol before electrophoresis. Samples in lanes 7-10 were electrophoresed under non-reducing conditions.
Lanes 1, 6: Molecular weight standards: M_r (kDa) indicated at left; lanes 2, 7: Vaccine Lot 2; lanes 3, 8: Vaccine Lot 5; lanes 4, 9: Vaccine Lot 8; lanes 5, 10: *S. sonnei* parent strain 53-G

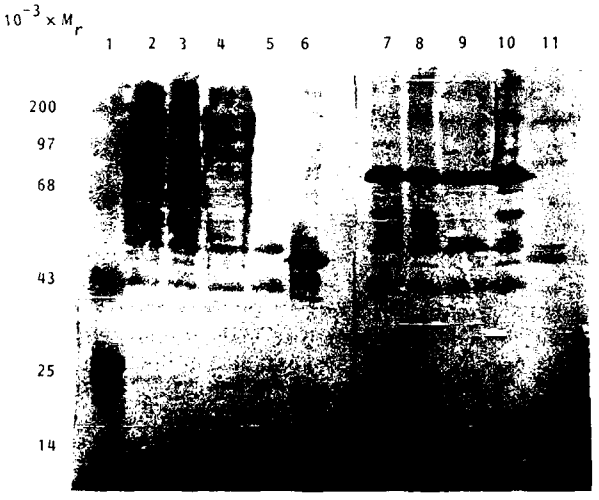

Figure 4 Western blot analysis of bacteria in vaccine lots #2, 5 and 8. Vaccine lot organisms and parental strains were separated on 12% gels, blotted to nitrocellulose, and probed with the respective rabbit antisera. Blots were developed with alkaline phosphatase-labelled goat anti-rabbit antiserum and visualized with naphthol AS MX phosphate and Fast Red TR. Lanes 2-6 developed with anti-*S. sonnei* antiserum; lanes 7-11 developed with anti-*S. typhi* antiserum.
Lane 1: M_r standards; M_r in kDa indicated; lanes 2, 7: Vaccine Lot 2; lanes 3, 8: Vaccine Lot 5; lanes 4, 9: Vaccine Lot 8; lanes 5, 10: *S. typhi* Ty21a; lanes 6, 11: *S. sonnei* 53-G

Western blots visualizing immunobinding bacterial proteins are shown in *Figure 4*. *Figure 4* shows that there is some cross reaction between *S. typhi* and *S. sonnei* by specific antisera (Lanes 5, 6, 10 and 11). There appear to be differences in the strength of immunobinding reactions when Ty-Shig Lots 2 and 5 are compared with Lot 8 (Lanes 2 and 3, *versus* 4, and Lanes 7 and 8 *versus* 9). Cell counts for these extracts were all approximately the same (*Table 1*), so the differences cannot be attributed to that parameter.

Figure 5 is a Western blot using vaccine pre-immunization or postimmunization sera. There seems to be more activity as seen by increased staining in the 30 day postimmunization sera in both cases, than in the pre-bleed sera. Most of this activity is in the higher molecular weight regions (above 68 kDa). Western blots of the IgG response of volunteers receiving the protective Ty-Shig Lot 2 vaccine and non-protective Lot 8 vaccine are shown in *Figure 6*. As in *Figure 5*, there is a somewhat greater degree of activity in the higher molecular weight region comparing pre-bleeds and serum from postimmunized patients. However, comparing the postimmunized patients sera for all three vaccine lots, there appear to be no distinct observable IgG reaction which distinguishes between the non-protective Lot 8 and the two protective lots of vaccine, 2 and 5. Therefore, looking for differences in vaccine lots by Western blot

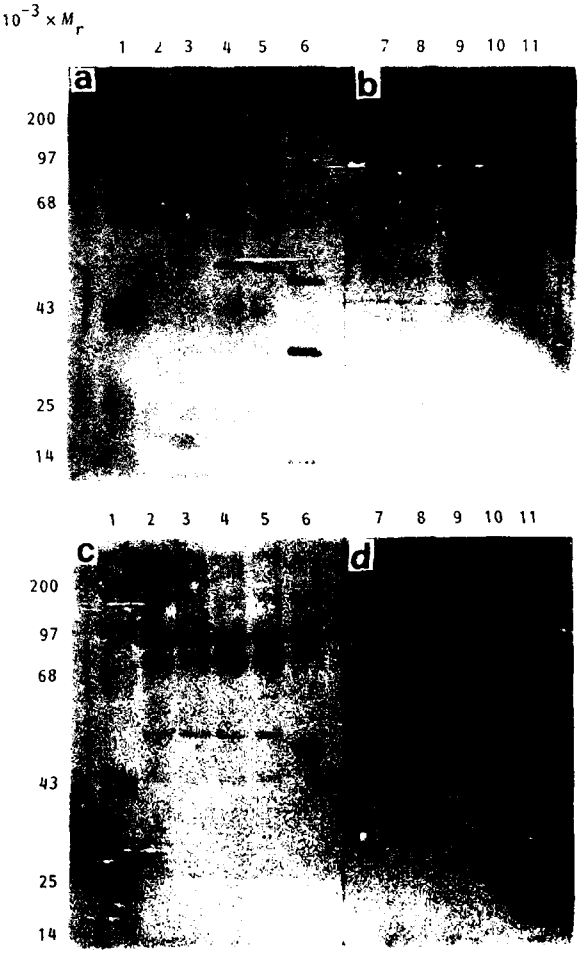

Figure 5 Western blots of human volunteers immunized with Lot #5 vaccine and orally challenged with *S. sonnei*. Vaccine and parental organisms were separated on 12% gels, blotted to nitrocellulose, probed with the indicated antiserum from the volunteers, developed with alkaline phosphatase-labelled goat anti-human antiserum, and visualized as described. (a) Lot 5 vaccine recipient, pre-bleed; (b) Same volunteer, 30 days postimmunization; (c) Lot 5 recipient, pre-bleed; (d) Same as c, 30 days postimmunization.
Lane 1: M_r standards; M_r in kDa indicated; lanes 2, 7: Vaccine Lot 2; lanes 3, 8: Vaccine Lot 5; lanes 4, 9: Vaccine Lot 8; lanes 5, 10: *S. typhi* Ty21a; lanes 6, 11: *S. sonnei* 53-G

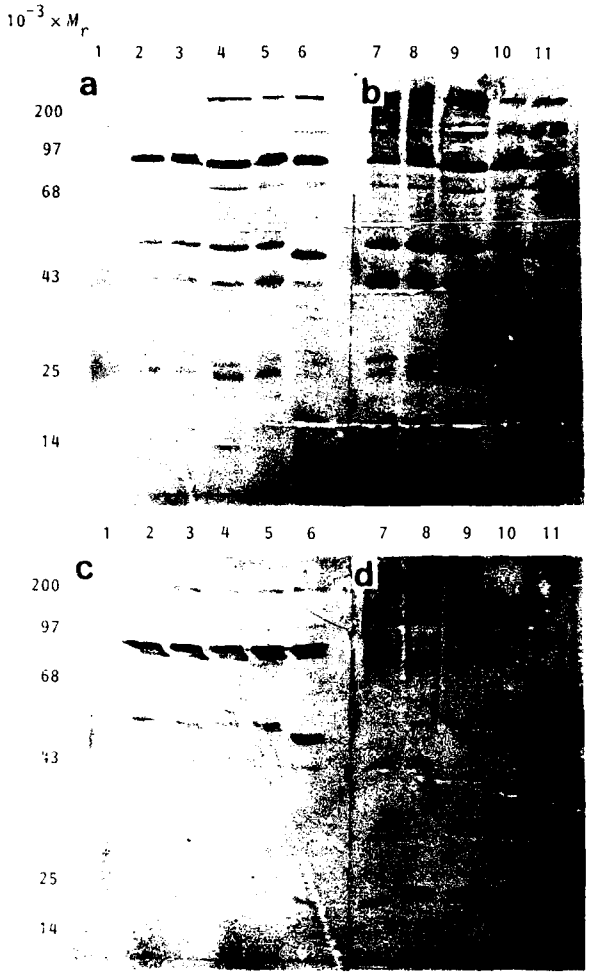

Figure 6 Western blots of human volunteers immunized with protective (Lot 2) and non-protective (Lot 8) hybrid vaccines. Procedures were as in Legend to *Figure 3*. (a) Volunteer receiving Lot 2 vaccine, pre-bleed; (b) same subject as a, 28 days postimmunization; (c) Volunteer receiving Lot 8 vaccine, pre-bleed; (d) Same as c, 28 days postimmunization.
Lane 1: M_r standards; M_r in kDa indicated; lanes 2, 7: Vaccine Lot 2; lanes 3, 8: Vaccine Lot 5; lanes 4, 9: Vaccine Lot 8; lanes 5, 10: *S. typhi* Ty21a; lanes 6, 11: *S. sonnei* 53-G

using specific polyclonal antiserum does not detect differences in immunoblot proteins of the vaccinating bacteria.

All three vaccines protected from infectious *Shigella* and *Salmonella* challenges as shown in *Table 2*. In this small study, immunized mice had survival rates of greater than 87.5%. The challenge strains were effective in killing 100% of the non-immunized mice. Oral immunizations and challenges were not used to demonstrate vaccine efficacy since this route of inoculation will not result in a productive infection in the mouse.

Discussion

The criteria listed in *Table 1* are the laboratory derived markers that are currently used to evaluate hybrid *Shigella* vaccines in the absence of an animal model. The data in *Table 1* show the fluid counts, freeze-dry counts, and percentage lactose positive cells to be about the same

Table 2 Protection of mice against *S. typhi* and *S. sonnei* challenge with vaccine strain 5076-1C

	Challenge strain[a]	
Vaccine (strain 5076-1C)	*S. typhi* Ty2 Mortality (no. dead/no. inoculated)	*S. sonnei* 53-G
Lot 2	2/16	0/16
Lot 5	2/16	0/16
Lot 8	0/16	0/16
Non-immunized mice	10/10	10/10

[a]Challenge dose *S. typhi* Ty2 = 10^4/0.5 ml suspended in 5% hog gastric mucin administered i.p.
Challenge dose *S. sonnei* 53-G = 10^2/0.5 ml suspended in 5% hog gastric mucin administered i.p.

for the three vaccines. Although somatic antigens were present for both *S. typhi* and *S. sonnei*, the flagellar H antigen was absent from the Lot 8 vaccine. The absence of a flagellar H reaction associated with the Lot 8 vaccine is puzzling since electron micrographs of bacteria in Lot 8 showed flagella associated with the cells. However, Gilman *et al.* found in volunteer studies that the presence of anti-H antibodies was associated with a lower illness level than in controls with no H antibody titre[8].

A non-motile strain of *S. typhi* Ty 2 which had no serum H antibody response, but which did possess a good O and Vi antibody response failed to provide protection against typhoid fever[10]. If H antigens are not involved in the protective immune response, their presence may be a marker for an as yet unidentified antigen. However, recovery of only 24% of Lot 8 organisms after freeze-drying may indicate that other structural damage to the organisms, in addition to loss of viability, resulted from this process. This may in part explain the near total loss of H antigen reactivity in these organisms. Pre-freeze-dry H antigen agglutination tests were not performed so this question remains unanswered. Another possibility is that passage of the Lot 8 master seed may have selected for a hybrid variant that was deficient in H antigen, or had an H antigen binding site that no longer reacted with the antiserum. This variant may also be more fragile and susceptible to loss of viability on freeze-drying. Two plasmid forms were identified by restriction analysis.

All three plots examined had a majority of the plasmid form that is lactose positive and Form 1 antigen positive. This observation indicates that the three lots had the same potential for producing the Form 1 antigen. However, since Lot 8 failed to protect humans against challenge, plasmid analysis would not be considered a reliable predictor as to whether the vaccine will be protective. If plasmid analysis indicated that there was a substantial departure in the plasmid profile from the protective Lot 5 vaccine, this would be indicative to not using a particular vaccine candidate. It is intuitively apparent that the majority of the organisms in a vaccine lot should contain the plasmid form which is lactose positive and which produces the *S. sonnei* Form 1 antigen.

Mouse protection data correlated with agglutination data in that somatic antigens were expressed and stimulated an immune response in mice (*Table 1*). However, the data clearly show that the mouse protection assay did not correlate with human challenge studies. As reported, Lots 2 and 5 protected humans against infectious *Shigella* challenge while Lot 8 did not[2]. No

data are presented concerning a challenge with infectious *Salmonella*, or serologic examination of body fluids for the presence of antibody to *Salmonella*. Data from the mouse protection assay is important because it demonstrates that the *Salmonella* and *Shigella* somatic antigens are present on the host cell and can cause a protective immune reaction to develop in mice inoculated by the IP route. Clearly, the mouse protection assay is of limited use for demonstrating vaccine efficacy in humans, who are vaccinated and challenged orally. These observations, taken with the differences seen in the freeze-drying data from the three vaccine lots is an indication that the organisms of Lot 8 changed in some basic way. However, all the vaccine lots had identical PAGE profiles. When serum IgG was visualized by western blot analysis, no reliable predictor of vaccine efficacy was noted. But, there was a somewhat heavier degree of staining in the postimmunization sera of volunteers than in their pre-bleed sera. There were also qualitative differences when the three vaccine lots were blotted. Clearly, pili (17000 M_r) are present as seen by electron microscopy. But, the antiserum used in this study is not specific enough to detect differences of this kind. There are no differences seen in the 17000 M_r region by either SDS-PAGE or western blot. A monoclonal antibody specific for the *Salmonella* pili protein would help to answer this question, but this awaits development. So, there seems to be some serum IgG response occurring, though this response is not a predictor of vaccine efficacy since Lot 8 was not protective in human volunteers.

Electron microscopic examination of the organisms from each of the three vaccine lots shows that while flagella are present in all three instances, no pili were present in the non-protective Lot 8 vaccine, whereas pili were present in vaccine Lots 2 and 5 (*Figure 2*). Incubation of bacteria from Lots 5 and 8 with rabbit anti-H antisera followed by colloidal gold-tagged Protein A and visualized by electron microscopy showed that the H antigen reaction was confined to the flagella of Lot 5 but not Lot 8 (data not shown)[11,13].

Pili have been shown to be sources of attachment for other members of the Enterobacteriaceae[1,2]. Hanson found in Sprague Dawley rats that pili bound to both the upper and lower small intestines of these animals[9]. Furthermore, it has been shown that pili-mediated adhesion can be blocked by D-mannose. Evans *et al.* and Evans and Evans have described plasmid-encoded pili antigens that are colonization factors in *E. coli*[4,5]. Beachy has shown that piliated bacteria can adhere to mucosal surfaces[1]. We have shown that the pili observed were products encoded by the *Salmonella* host cell. It is conceivable that the lack of pili would result in direct passage of the vaccinating organisms through the GI tract with no colonization. Rapid clearance of the organism would thus prevent an immune response. Therefore, based on the electron microscopic and H antigen evidence we feel that the presence of pili and a substantial H antigen reaction are the important features in the success of the hybrid Ty Shig vaccines.

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